AD_____

Award Number: W81XWH-04-1-0360

TITLE: Rho GTPase Involvement in Breast Cancer Migration and Invasion

PRINCIPAL INVESTIGATOR: Kaylene J. Simpson, Ph.D.

CONTRACTING ORGANIZATION: Harvard Medical School
Boston MA 02115-6027

REPORT DATE: March 2007

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
01-03-2007	Annual Summary	01 Mar 06 – 28 Feb 07

4. TITLE AND SUBTITLE
Rho GTPase Involvement in Breast Cancer Migration and Invasion

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-04-1-0360

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Kaylene J. Simpson, Ph.D.

E-Mail: kaylene_simpson@hms.harvard.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Harvard Medical School
Boston MA 02115-6027

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT:
Using a high throughput small interfering RNA approach (siRNA) I have screened 1081 human genes (kinases, phosphatases and a library of migration-related genes) using an automated wound healing assay to identify genes that regulate cell migration using the normal mammary epithelial cell line MCF10A. Genes were classified into hit bins based on motility (either accelerated or impaired migration) and metabolism (measured by Alamar Blue). Focusing in greater detail on the 101 genes that accelerate migration, extensive validation assays were performed reducing the set of high confidence genes to 31. Signaling network analysis of these genes reveals the β-catenin pathway is heavily involved. Analysis of published breast tumour microarray data has directed our immediate priorities towards a number of genes that were hits in our screen and identified in the arrays. The signaling pathways regulating cell migration of these new targets remains to be elucidated.

15. SUBJECT TERMS
Breast cancer, metastasis, tumour biology, Rho GTPases, cell signalling, integrins, siRNA screening, wound healing

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			USAMRMC
U	U	U	UU	22	**19b. TELEPHONE NUMBER** *(include area code)*

Table of contents

Cover…… 1

SF298…… 2

Introduction…… 4

Body…….. 4

Key Research Accomplishments…… 7

Reportable Outcomes……. 7

Conclusion …… 8

References…….. 8

Appendices…….. 9

Introduction

The third year of this project focused on gene discovery, identifying novel regulators of cell motility using a high throughput small interfering RNA (siRNA) screening approach. The objective was to identify genes that regulate motility in a normal mammary epithelial cell line, particularly in the scenario where loss of gene expression results in a significantly motile cell. siRNA screening is currently the cutting edge of mammalian siRNA technology (1), is still extremely expensive, requires access to robotic facilities, high throughput microscopy, extensive data storage and intensive bioinformatics analysis. Within the Department of Cell Biology at Harvard Medical School I have gained access to all the tools necessary to successfully undertake such research.

Body – progress report

AIM 4: Develop high throughput siRNA screening technology to take an unbiased approach to identify novel genes that regulate motility and invasion of breast cells.

To achieve the sub-categories of this Aim, I developed the conditions to robotically transfect, wound and image cells in a 96 well format. The siRNA screen comprised 1081 individual genes that were derived from the human protein and lipid kinases (577 genes), phosphatases (192 genes) and a custom (migration-related) library of 312 genes that were hand selected to encompass extended family members of genes that have been implicated in cell migration and invasion in cell lines and a range of tumour types of different origins. The primary screen used Dharmacon *SMART*pools, an aggregation of 4 individual sequences to each gene and centred on the normal mammary epithelial cell line, MCF10A and the classic wound healing assay to measure cell motility (2). These cells form a classic cell-cell contacted cobblestone epithelial cell monolayer and are non-motile unless stimulated to move by addition of EGF. We established that 12 hours of wound healing was sufficient to distinguish accelerated and impaired migration from controls and developed an automated numerical measure of cell motility. In addition, a visual call was made for each wound and in some cases was used to over-ride the numerical score. The impact of the siRNA on general cell metabolism was measured in parallel using an Alamar Blue reduction assay. The phenotypic range of the assay and functional classifications are indicated in Figure 1.

Aim 4.1 – complete screening of the migration-related siRNA library initiated in the pilot study phase.
Aim 4.2 – screen the human protein kinase and phosphatase siRNA libraryes using the wound healing approach.
These 2 Aims have been completed and the data has been merged to present an overview of the entire screen, encompassing the 3 different libraries.

Collation of the data from the kinase, phosphatase and custom library screen in MCF10A cells is shown in Figure 2. Hits were binned into 3 categories, accelerated migration (closing bin) comprised 101 genes (9.3% of total), impaired migration (Open bin) comprised 202 genes (18.7% of total) and impaired cell metabolism (low alamar bin) comprised 154 genes (14.2% of total). These data are the collation of 3 independent screens performed in duplicate, with overall reproducibility of 88%. The distribution of the hit bins is indicated for each library (Figure 2B). It is interesting to note that 75% of the phosphatase genes did not evoke a motility response in these cells.

To validate the phenotype of the *SMART*pools and eliminate potential false phenotypes, we screened 2 of the 4 individual sequences that constitute the pool. For 39% of genes the phenotype of both individual

sequences matched the *SMART*pool while 36% showed 1 of 2 (Figure 2C). It was difficult to reconcile those genes in which only 1 sequence matched the pool, therefore we chose to focus in greater detail on the 101 genes that accelerated migration and repeated the screen using re-synthesised *SMART*pool and 4 individual sequences on the same plate. We identified 31 high confidence genes that showed either 3 or 4 of the 4 individuals matching the *SMART*pool phenotype and a further 32 lower confidence genes that showed 2 of the 4 matching (Figure 3A). Morphologically we observed tight concordance between wells as shown by knockdown of RHOA (Figure 3B). One of the hallmarks of tumour success and progression to metastatic sites is extravasation of the cells from the primary tumour and migration to secondary sites (3). Typically *in vitro* this is observed by loss of cell-cell contact and an elongated morphology termed an epithelial to mesenchymal transition (3). It is interesting to note that we observed 14/101 closing genes that have undergone such striking morphological changes, with a high proportion (5 of the 11) falling into the high confidence 4 of 4 concordance category (Figure 3Ci). Of the remainder the majority maintain strong cell-cell contact and continue to move as a sheet, seemingly requiring support from surrounding cells to maintain motility (Figure 3Cii).

The extent of gene knockdown was established for the kinase and phosphatase genes using branched DNA (bDNA) technology (Genospectra) in collaboration with Dharmacon RNA technologies. This represents a significant undertaking and one that remains elite within the RNAi screening arena. Knockdown was established for 762 of the 769 kinase and phosphatase genes and was performed in parallel with wound healing. It was striking to see that a significant proportion, 220 genes (29%) showed greater than 80% knockdown, while overall, 393 genes (52%) showed knockdown greater than 60% (Figure 4). 126 genes recorded a knockdown value of <20% and of these, 80 genes were previously undetectable by bDNA in baseline detection analysis, reflecting the limited sensitivity of the assay for genes that may be poorly expressed, since this assay is measuring direct quantities, not amplifying the signal as for PCR-based methods. A further 115 genes were not scored due to various technical issues. Interestingly, we did not observe any significant enrichment of knockdown in any of the hit categories (Figure 3, see overlay), but within each phenotypic category we observed a similar knockdown distribution, that is, approximately one-third of the genes in each group were knocked down >70%.

Aim 4.3- collate data from all screens, segregate into functional categories and analyse the data for pathway linkages using bioinformatics approaches.

Our first approach to informatically mine this data was to interrogate 3 independent databases (NIH DAVID, Bioknowledge library, Gene Sifter) using broad gene ontology terms. We chose terms that relate directly to the biological process of cell motility, including Cell Motility, Cell Migration, Cell Adhesion and Chemotaxis, and those that are involved in cell structural regulation such as cytoskeleton, lamellipodium and focal adhesion. In addition, we looked at general processes such as cell death, proliferation and cell cycle. There was significant overlap between these terms and we found that many of the hit genes had been previously implicated in the literature as having some role in cell migration. In order to identify new players in the migration field, we collated the high confidence genes that accelerated or impaired migration (not the low alamar sub group) and had no association with cell migration terms and worked backwards to determine the binding partners of these proteins. There are several interactome databases and we chose to use BOND (Biomolecular Object Network Databank), a database that has an exhaustive and frequently updated compilation of mammalian interaction data. Of the 81 genes that we used as bait, we identified 160 unique interactions, of which 37 had been screened in our assay. We are now working towards identifying whether these interactions are real and viable to follow.

To investigate signalling pathways we have used the Ingenuity software package and queried the network linkages of the genes in the closing, open and low alamar hit bins. The top network that incorporates many of the hits from each category revolves around the β-catenin signalling pathway which is a critical pathway for many aspects of development and disease (4). Lesser pathways include various growth factor networks such as EGF, PDGF and VEGF. This is the first step towards identifying new migration pathways and will require biochemical validation.

To further define priority candidates and link direct disease associations we collated the microarray data from 3 recent studies that compared different tumour subclasses, most notably the aggressive basal tumours with non-basal tumours and ER^-PR^- tumours with ER^+PR^+ tumours (5, 6, 7). Focusing on the genes that were up- or down-regulated in at least 2 of the 3 studies revealed 53 genes (10 Closed, 27 Open and 16 Low Alamar) that were up-regulated in basal/basal-like tumours and 37 genes (7 Closed, 22 Open and 8 Low Alamar) that were down-regulated in basal/basal-like tumours. Future studies will be directed towards pathway analysis of these genes and subsequently focusing on a subset that have most clinical relevance.

Aim 4.4 Develop tertiary assays to further subdivide functional categories and follow up critical candidates at a biochemical level.
Several different approaches were used to sub-classify the genes in different hit bins.
I attempted to set up high throughput time-lapse video microscopy to image cells during the 12 hour wound closure period. We encountered many technical obstacles, particularly as optimal imaging required the use of glass bottom plates and fluorescent tagged cell lines. We found the cells did not migrate as efficiently on glass bottomed plates and we were unable to generate fluorescent tagged lines that emitted sufficient fluorescence for detection over time. We tried various vital dyes but all were toxic to the cells over the 12 hour wounding period.

High content staining of the closing genes with the cell-adhesion markers E-cadherin and N-cadherin was initiated but completed. Whilst the staining worked, I was unsuccessful in developing a computational program that was fully quantitative, particularly as we observe delocalisation of these proteins in response to altered cell morphology and migration and not loss of expression.

We aimed to establish which low alamar sub-class genes were binned as a result of loss of cell adhesion, altered metabolism or cell death. To address the cell death aspect we screened the 154 low alamar genes using MCF10A cells expressing Bcl2. Interestingly we observed only 9 genes in which the low alamar phenotype was rescued (NCAM1, MSX1, PLAUR, PVR, RABGEF1, SORBS1, MAPKAPK, NRCAM, TGFB1I1), suggesting these genes may have a role in cell death in the MCF10A cells.

We chose several high confidence closing genes (ADCK4, PTPRO, MAPK14, CTNNB1, CTNND1, CTNNA1, PPP1R1B) as candidates for longer term stable knockdown analysis using a short hairpin RNAi approach (shRNA- OpenBiosystems). There are 4 or 5 different constructs per gene and we are currently characterising the migration phenotype for each line. We will then analyse the migration phenotype in 3D, embedding the cells in basement membrane (Matrigel), a strong predictor of behaviour *in vivo*. More extensive biochemical analysis will begin once we determine the 2D migration phenotype is recapitulated in 3D.

Aim 4.5 – identify genes that interact with RHOA and RHOC.

This aim has not yet been addressed.

Key Research accomplishments

- Screened 1081 genes using a high throughput siRNA approach
  - Established the extent of knockdown for the kinase and phosphatase genes
  - Identified a subset of genes that migrate with an altered 'mesenchymal-like' morphology
  - Identified genes that migrate without losing cell-cell contact
  - Identified the β-catenin signalling pathway as a major migration signalling node
  - Identified a number of genes as future candidates for analysis that have breast carcinoma disease association
- Generating stable knockdown cell lines of key candidate genes using a lentiviral shRNA approach.

Reportable outcomes

Publications

Bellovin DI, **Simpson KJ**, Danilov T, Maynard E, Rimm DL, Oettgen P and Mercurio AM. Reciprocal Regulation of RhoA and RhoC Characterizes the EMT and Identifies RhoC as a Prognostic Marker of Colon Carcinoma. *Oncogene 2006;* 25(52):6959-6967; May 22, epub

Oral presentations

Women's Leadership Board, Kennedy School of Government, Harvard University. Stem cells and breast cancer – exploring the possibilities. May 4, 2006.

Sanofi Aventis. Cambridge Massachusetts. Identifying genes that regulate cell motility using high throughput siRNA technology. May 11, 2006.

VAV Program Project Grant meeting, Harvard Medical School. An siRNA screen for genes regulating cell motility. October 5, 2006.

American Society of Cell Biology, Annual meeting, San Diego. Acceleration and suppression of human epithelial cell migration by siRNA. December 9, 2006.

Walter and Eliza Hall Institute, Australia. Acceleration and suppression of epithelial cell migration using a high throughput siRNA approach. February 23, 2007.

Abstracts

Simpson KJ, Selfors LM, Bui J, Reynolds A, Leake D and Brugge JS. American Society of Cell Biology, Annual meeting, San Diego. Acceleration and suppression of human epithelial cell migration by siRNA. December 9, 2006.

Training

- Full time supervision of a technical assistant
- Supervision of a rotating Graduate student
- Manuscript revisions both independently and in conjunction with Dr. Brugge
- Continued involvement in organization of the Longwood area mammary gland meeting

Current collaborations
Dr. Devin Leake and Angela Reynolds, Thermofisher (formerly Dharmacon RNA technologies). Quantitation of siRNA knockdown using a high throughput screening approach.

Dr. Scott Snapper and Dr Anna Lyubimova, Massachusetts General Hospital Department of Medicine, Boston. Investigating the role of N-WASP in cell motility.

Dr. Alexei Degterev, Tufts University, Boston. Characterisation of small molecule inhibitors with respect to inhibition of cell motility in breast carcinoma cells.

General comments
A no-cost extension has been granted to this award that will allow outstanding experiments necessary for the completion of the manuscript to be finalised. Some of the data generated in this past year was used to gain NIH funding as we are members of the Gene Expression group of the Cell Migration Consortium.

Conclusions
RNAi screening remains fairly exclusive technology. We have made significant progress over the course of the past year and gone through extensive validations to be confident of out hits. We have identified a number of candidate genes that either positively or negatively regulate epithelial cell migration. We have chosen to focus on potential tumour suppressor genes, those in which loss of expression results in increased migration potential. Informatics analysis of a subset of the breast tumour literature has provided a direct focus on potential candidates for future biochemical studies

References
1. Moffat J, Sabatini DM. Building mammalian signalling pathways with RNAi screens. Nat Rev Mol Cell Biol. 2006; 7(3):177-187.

2. Valster A, Tran NL, Nakada M, Berens ME, Chan AY, Symons M. Cell migration and invasion assays. Methods. 2005; 37(2):208-15.

3. Gupta GP and Massague J. Cancer Metastasis: Building a framework. Cell 127(4):679-695.

4. Clevers H. Wnt/β-catenin signalling in development and disease. Cell 127(3):469-480.

5. Farmer P, Bonnefoi H, Becette V, Tubiana-HUlin M, Fumoleau P, Larsimont D, MacGrogan G, Bergh J, Cameron D, Goldstein D, Duss S, Nicoulaz A, Brisken C, Fiche M, Delorenzi M and Iggo R. Identification of molecular apocrine breast tumours by microarray analysis. Oncogene 2005, 24(29):4660-71.

6. Richardson Al, Wang ZC, De Nicolo A, Lu Z, Brown M, Miron A, Liao X, Iglehart J, Livingston DM and Ganesan S. X chromosomal abnormalities in basal-like human breast cancer. Cell 2006, 9(2);121-32

7. Chin K, DeVries S, Fridlyand J, Spellman PT, Roydasgupta R, Kuo W, Lapuk A, Neve RM, Qian Z, Ryder T, Chen F, Feiler H, Tokuyasu T, Kingsley C, Dairkee S, Meng Z, Chew K, Pinkel D, Jain A, Ljung BM, Esserman L, Albertson DG, Waldman FM and Gray JM. Genomic and transcriptional aberrations linked to breast cancer pathophysiologies. Cancer Cell 2006, 10(6):529-41.

Appendices
Bellovin et al., 2006, Cancer Research manuscript

Supporting data
Figure 1 - Phenotypic range of the wound healing assay.

Figure 2 - Schematic representation of the siRNA screen of the kinase, phosphatase and custom (migration-related) genes.

Figure 3 - Further validation of the closing set of 101 genes.

Figure 4 - Distribution of the degree of knockdown of the kinase and phosphatase *SMART*pools.

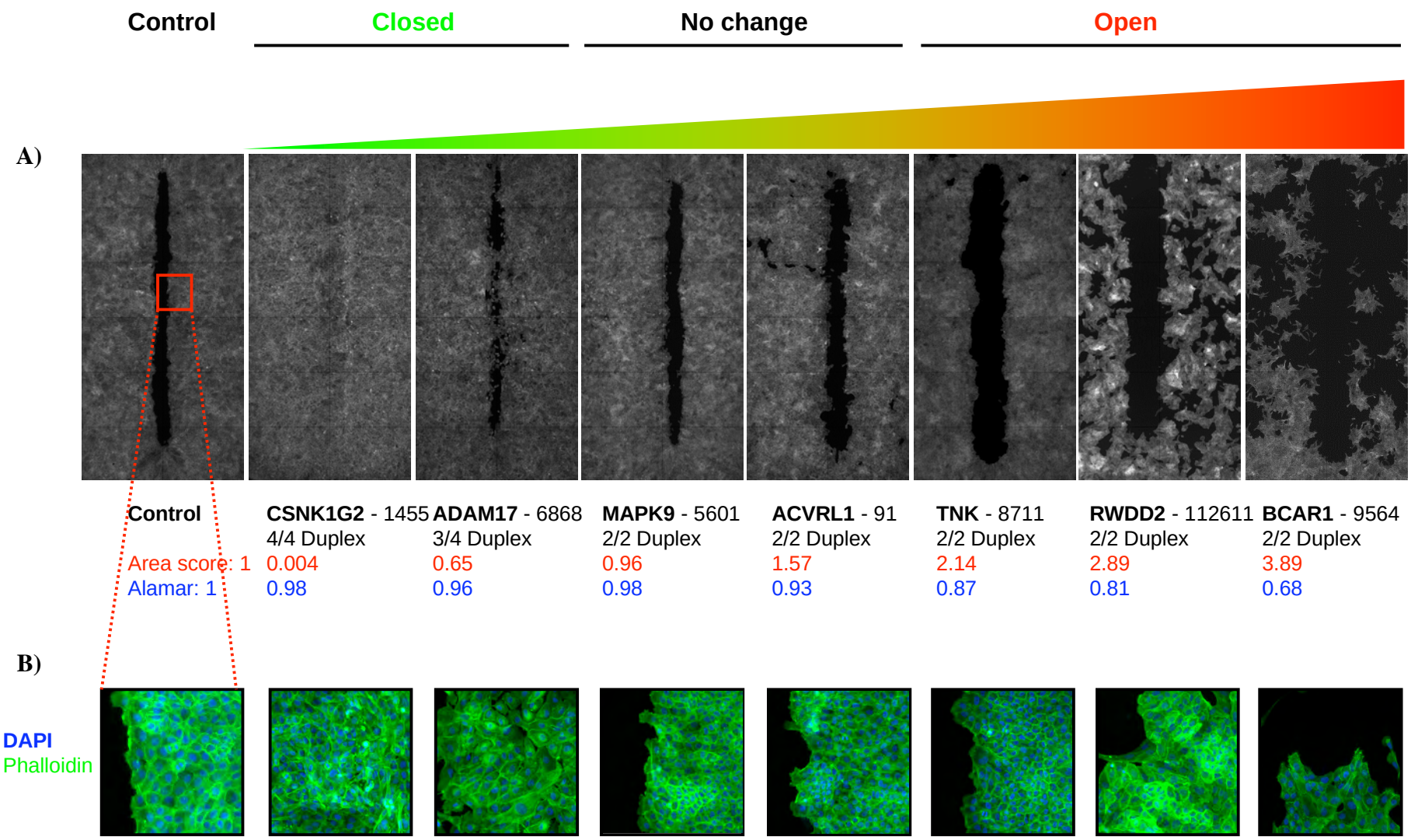

Figure 1 - Phenotypic range of the wound healing assay. A) Hits were classified on the basis of the numerical Area score (scores indicated in red) and a visual score, both normalised to the control. Genes that accelerated migration (closed bin) scored <0.75 and those that impaired migration (Open bin) scored >2.0. Genes that scored <0.8 in the Alamar blue assay (scores indicated in blue) were grouped into a low alamar subclass. Each gene is listed alongside its Entrez Gene ID and the phenotypic duplex concordance score is shown. B) Images were captured at high resolution which enables detailed analysis of the actin cytoskeleton (via Phalloidin staining) and general cell morphology as shown.

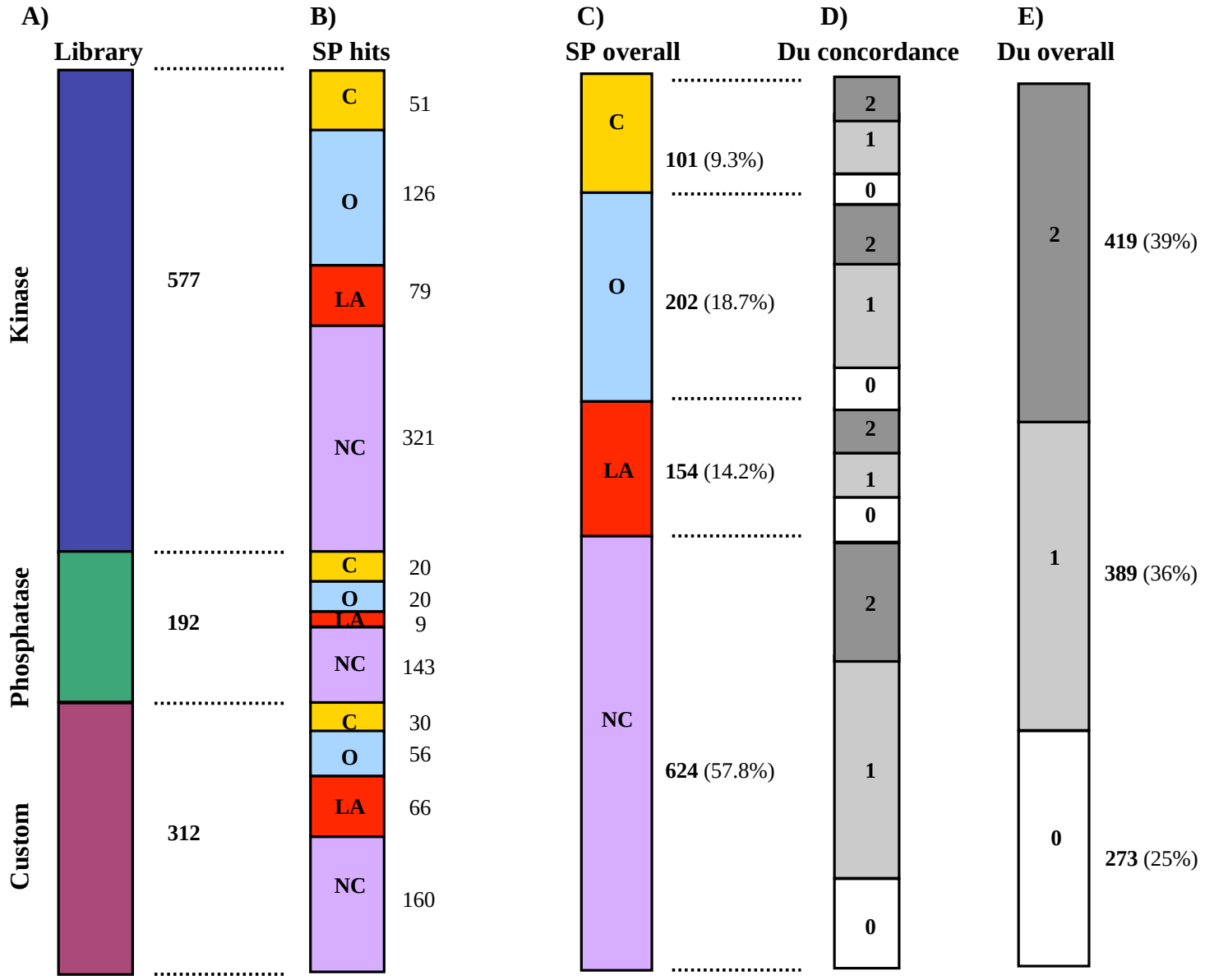

Figure 2- Schematic representation of the siRNA screen of the kinase, phosphatase and custom (migration-related) genes. A) *SMART*pools. B) Classification of genes into hit bins, Closed (C), Open (O), Low Alamar (LA) and No Change (NC) and their distribution within each library. Overall distribution of hits (C) and the phenotypic concordance of the 2 individual sequences matching the SMARTpool (D). If both sequences matched they scored a 2, 1 sequence scored 1 and no phenotypic concordance scored 0. The overall distribution of phenotypic duplex concordance is shown in part E.

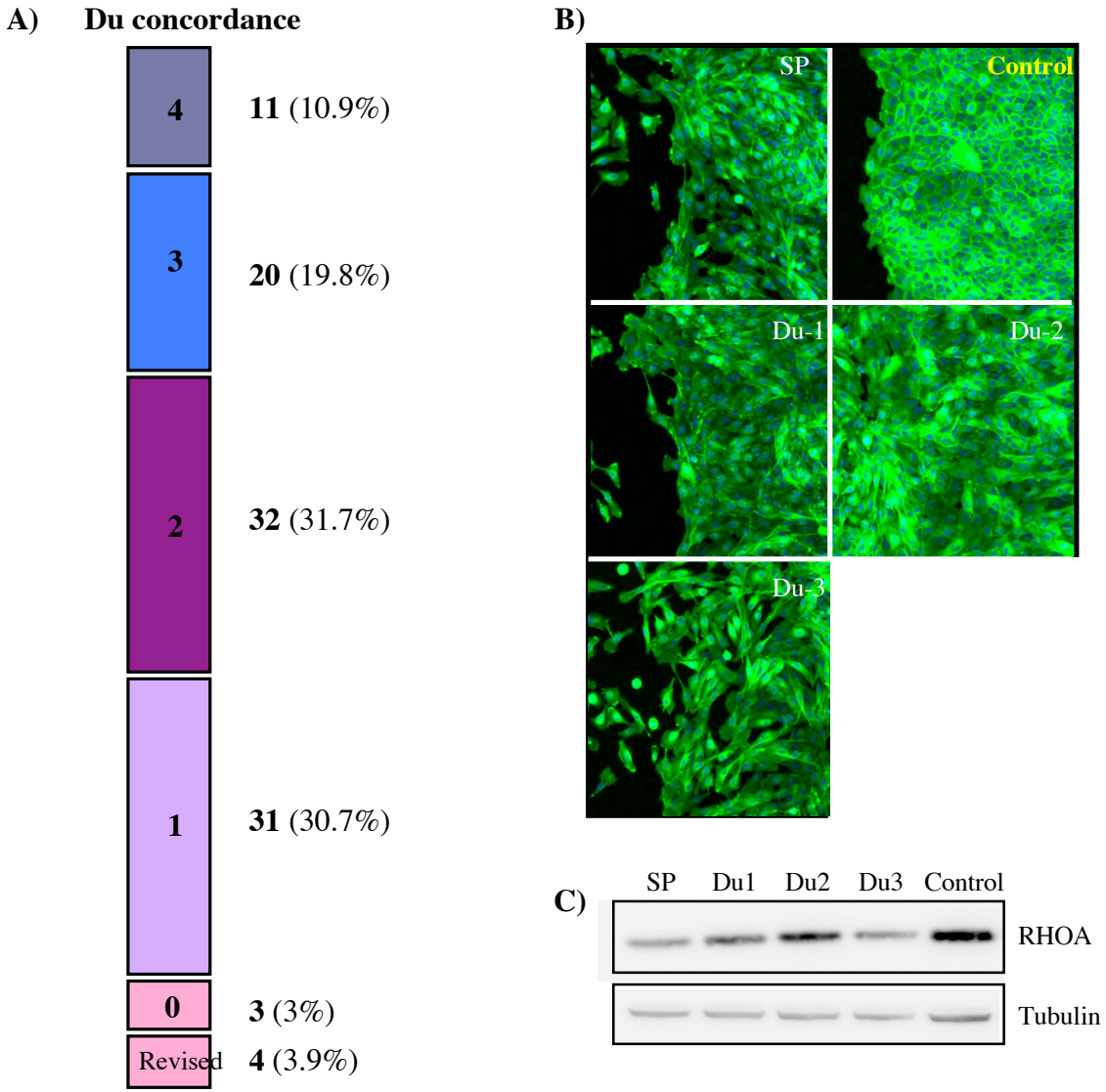

Figure 3 - Further validation of the closing set of 101 genes. Phenotypic concordance after screening the 4 individual sequences that comprise the *SMART*pool (A). Highest confidence can be placed in those that had 3 or 4 of the sequences phenotypically matching the pool. Morphological concordance was observed for the *SMART*pool and individual sequences (Du1, 2, 3) as shown after knockdown of RHOA (B). Western analysis shows the RHOA *SMART*pool and individual sequences efficiently targeted the RHOA gene. High resolution imaging of (Ci) knockdown of CTNND1 results in an altered morphology reminiscent of an epithelial to mesenchymal transition and (Cii) increased motility without loss of cell-cell contact for PTPRO and (Ciii) mock-transfection control.

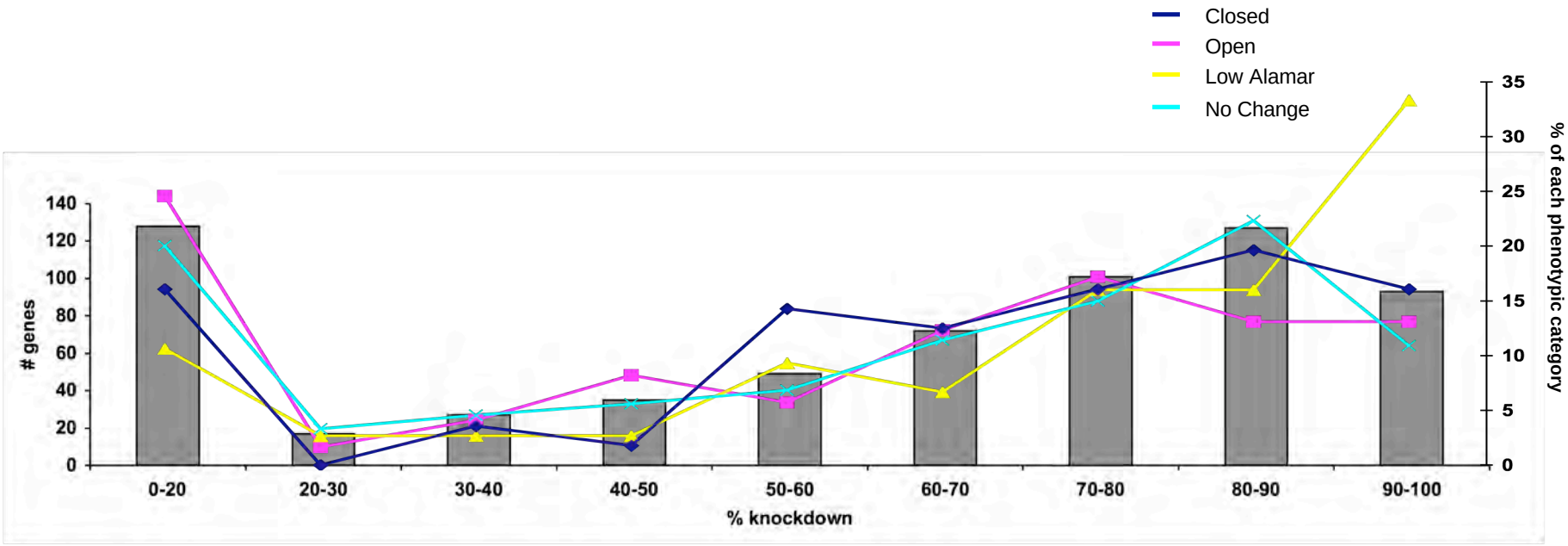

Figure 4 - Distribution of the degree of knockdown of the kinase and phosphatase *SMART*pools. Knockdown was grouped into percentile bins and shows that 61% showed knockdown of greater than 60% and significantly, 34% of genes knocked down greater than 80%.
The distribution of each hit classification is shown within each knockdown percentile. Statistical analysis using the Kruskal-Wallis non-parametric ANOVA shows no significant enrichment for knockdown within any phenotypic class.

Oncogene (2006) 25, 6959–6967

www.nature.com/onc

ORIGINAL ARTICLE

Reciprocal regulation of RhoA and RhoC characterizes the EMT and identifies RhoC as a prognostic marker of colon carcinoma

DI Bellovin[1], KJ Simpson[2], T Danilov[3], E Maynard[3], DL Rimm[4], P Oettgen[3] and AM Mercurio[1,5]

[1]*Division of Cancer Biology and Angiogenesis, Department of Pathology Beth Israel Deaconess Medical Center and Harvard Medical School, Boston, MA, USA;* [2]*Department of Cell Biology, Harvard Medical School, Boston, MA, USA;* [3]*Division of Preventive Cardiology, Beth Israel Deaconess Medical Center and Harvard Medical School, Boston, MA, USA and* [4]*Department of Pathology, Yale University Medical School, New Haven, CT, USA*

Understanding how RhoC expression and activation are regulated is essential for deciphering its contribution to tumorigenesis. Here, we report that RhoC expression and activation are induced by the epithelial to mesenchymal transition (EMT) of colon carcinoma. Using LIM 1863 colon cancer cells, RhoC protein expression and subsequent activation were detected coincident with the loss of E-cadherin and acquisition of mesenchymal characteristics. Several Ets-1 binding sites were identified in the RhoC promoter, and evidence was obtained using chromatin immunoprecipitation that Ets-1 can regulate RhoC expression during the EMT. Interestingly, a marked decrease in RhoA activation associated with the EMT was observed that corresponds to the increase in RhoC expression. Use of shRNA established that RhoA inhibits and RhoC promotes post-EMT cell migration, demonstrating functional significance for their coordinate regulation. To assess the importance of RhoC expression in colon cancer, immunohistochemistry was performed on 566 colorectal tumors with known clinical outcome. The level of RhoC ranged from no expression to high expression, and statistical analysis revealed that elevated RhoC expression correlates with poor outcome as well as aberrant expression and localization of E-cadherin. These data provide one mechanism for how RhoC expression is regulated in colon carcinoma and substantiate its utility as a prognostic marker.
Oncogene (2006) **25,** 6959–6967. doi:10.1038/sj.onc.1209682; published online 22 May 2006

Keywords: RhoC; epithelial mesenchymal transition; colorectal carcinoma

Introduction

Elucidating the key events of carcinoma progression is critical for our understanding of the disease, as well as our ability to properly manage it. Consequently, the degree of cytodifferentiation of a carcinoma can provide important insight into the nature and behavior of the tumor. In general, loss of epithelial characteristics during tumorigenesis is associated with increased aggressiveness and poor prognosis. Such observations have led to the implication of epithelial to mesenchymal transition (EMT) as a mechanism of carcinoma progression (Thiery, 2002; Thompson *et al.*, 2005). EMT describes the trans-differentiation of epithelium into mesenchyme that occurs during embryonic development. Given that poorly differentiated carcinomas resemble mesenchymal cells, often exhibiting expression patterns associated with an EMT such as loss of E-cadherin and expression of mesenchymal proteins such as vimentin, this transition is used increasingly to explain the genesis of high-grade carcinomas (Thompson *et al.*, 2005). Indeed, proteins associated with the EMT are involved in tumor progression, often serving as inducers of a phenotypic change, enhancers of invasion and metastasis, or markers of a more foreboding patient outcome (Yang *et al.*, 2004; Moody *et al.*, 2005; Roy *et al.*, 2005). In addition, EMT may not be restricted only to high-grade tumors because a localized EMT may occur at the invasive front of more differentiated tumors to facilitate their egress into surrounding tissue. As such, the EMT can be a powerful approach for identifying molecules whose expression is associated with aggressive disease and that may be potential therapeutic targets.

A valuable model for studying the EMT process in tumor progression are LIM 1863 colon carcinoma cells, which grow as highly differentiated organoids in culture and exhibit a three-dimensional structure that recapitulates the colonic epithelium. In response to stimulation with tumor growth factor (TGF)-β and tumor necrosis factor (TNF)-α, these spheroids undergo a rapid conversion to a more mesenchymal, two-dimensional architecture that involves loss of E-cadherin, delocalization of p120ctn, and expression of mesenchymal proteins (Bates and Mercurio, 2003; Bellovin *et al.*,

Correspondence: Dr AM Mercurio, Department of Cancer Biology, University of Massachusetts Medical School, LRB-408, 364 Plantation Street, Worcester, MA 01605.
E-mail: arthur.mercurio@umassmed.edu
[5]Current address: Department of Cancer Biology, University of Massachusetts Medical School, Worcester, MA 01605, USA.
Received 13 January 2006; revised 31 March 2006; accepted 31 March 2006; published online 22 May 2006

2005). The consequence of this conversion is the acquisition of chemotaxis and the ability to survive as single cells. This transition appears to involve profound changes in the cytoskeleton in a manner associated with altered activity of multiple Rho GTPases. In this direction, we reported recently that, although excess RhoA can result in cellular transformation, the EMT process involves a suppression of RhoA activity, which is essential for the ability of these cells to migrate efficiently (Bellovin *et al*., 2005). These observations suggest that the contributions of RhoA to tumor initiation and progression may differ and that the regulation of RhoA activation is critical for these processes.

Our findings on RhoA activation and the EMT of colon carcinoma raise the possibility that RhoC, a closely related member of the Rho GTPase family, may be modulated in response to the EMT as well. This possibility is substantiated by reports that have demonstrated the correlation of RhoC expression with the progression and metastasis of several tumor types (van Golen *et al*., 1999; Clark *et al*., 2000; Shikada *et al*., 2003; Wang *et al*., 2004b). Moreover, a recent transgenic study using RhoC−/− mice concluded that this protein is not necessary for the development of mammary tumors but that it is essential for metastasis to the lungs (Hakem *et al*., 2005). The hypothesis can be derived from these findings that activated RhoC is a distinguishing characteristic of more aggressive tumors and that the EMT may be one mechanism for regulating RhoC function in cancer. However, the mechanisms by which RhoC transcription and GTPase activity are regulated during tumorigenesis remain to be determined.

We demonstrate here that RhoC expression and activation are induced during EMT of colon carcinoma and that its expression can be regulated by the Ets-1 transcription factor. Moreover, a reciprocal regulation of RhoA and RhoC activation characterizes the EMT and is essential for the migration of post-EMT cells. To assess the clinical relevance of these observations, we examined the expression of RhoC protein in 566 primary colorectal tumors. Increased RhoC expression correlates with a significant reduction in patient survival time, substantiating the use of RhoC as a prognostic marker and potential therapeutic target for colon carcinoma. Interestingly, RhoC expression also correlates with altered expression and localization of E-cadherin, providing further evidence that its expression is linked to an EMT process in colon carcinoma.

Results

RhoC is expressed and activated during EMT of colon carcinoma

The EMT of LIM 1863 organoids is coincident with a suppression of RhoA GTPase activity, which is necessary to enable the migration of cells that have lost E-cadherin expression (Bellovin *et al*., 2005). Although no significant change in the level of RhoA protein occurs in response to stimulation with TGF-β and TNF-α, the expression of RhoC protein was detected, beginning within 4 h of cytokine treatment and reaching maximal expression at 24 h (Figure 1a). This expression was detected using a novel polyclonal antibody generated against a peptide from the carboxy terminal region of RhoC, the specificity of which is confirmed in later experiments utilizing shRNA (Figure 3a and b). The induction of RhoC expression coincides with loss of E-cadherin (Figure 1a) and the acquisition of an adherent, motile phenotype (not shown). Moreover, a marked increase in RhoC mRNA occurs early during EMT, suggesting that the induction of RhoC expression may be the result of transcriptional activation (Figure 1b). The expression of RhoA mRNA, in contrast, does not increase in response to cytokine stimulation, consistent with the protein data (Figure 1b).

To determine whether RhoC is activated subsequent to its expression, the Rhotekin pulldown assay was used to assess the proportion of RhoC protein bound to GTP in LIM 1863 cells upon cytokine treatment. Indeed, significant RhoC activity is detectable 18 h after cytokine stimulation and is sustained until at least 36 h (Figure 1c). Interestingly, the increase in RhoC

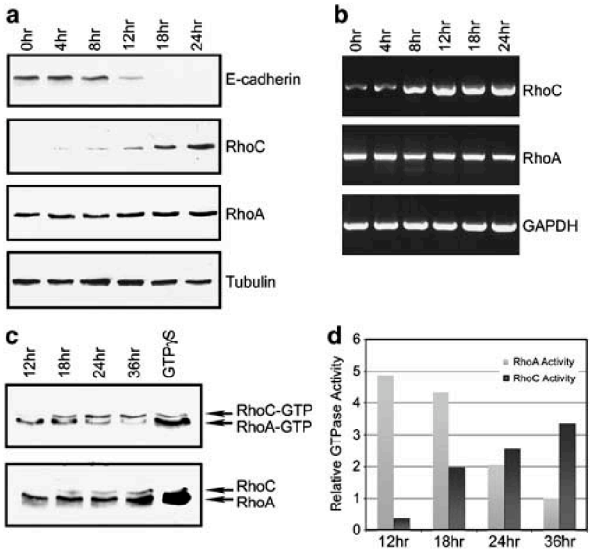

Figure 1 RhoC expression is regulated in an EMT-dependent manner. (**a**) Extracts from LIM 1863 cells stimulated with TGFβ and TNFα for the times indicated were blotted with Abs specific for either RhoC or RhoA (see Materials and methods). An E-cadherin immunoblot indicates timing of EMT, while tubulin levels validate the equivalent amounts of protein in each lane. (**b**) mRNA was isolated from LIM 1863 cells and RT–PCR specific for either RhoC or RhoA was performed. GAPDH RT–PCR demonstrates equivalent mRNA in all samples. (**c**) Extracts from stimulated LIM 1863 cells were assayed for their level of (active) RhoC-GTP and RhoA-GTP and for total expression of Rho proteins. An antibody that recognizes both proteins was used to compare activity and expression directly. (**d**) The blots in (**c**) were analysed by densitometry to obtain relative activation (active/total) as a function of time of cytokine stimulation. RhoA activity is represented in gray and RhoC activity in black bars. These data are representative of multiple, independent experiments.

activation occurs concomitantly with a decrease in RhoA activation (Figure 1d).

RhoC transcription is regulated by Ets-1 during EMT of colon carcinoma

An important issue that arises from these data is how RhoC expression is regulated during the EMT of LIM 1863 cells. Analysis of the RhoC promoter revealed multiple putative binding sites for the Ets-1 transcription factor (GGAA/T) (Woods *et al*., 1992). In fact, there are several regions containing clusters of three or more potential sites (Figure 2a). To define the role of Ets-1 in regulating the RhoC gene, two fragments encoding the proximal 280 and 990 basepairs of the RhoC promoter were cloned into the PGL3 luciferase reporter plasmid. The ability of Ets-1 to transactivate the RhoC promoter was then tested in cotransfection studies (Figure 2a). Whereas cotransfection with the short construct led to a modest increase in transactivation (2.6-fold induction), cotransfection with the larger construct resulted in a 5.3-fold induction of the reporter (Figure 2b). Further analysis of this larger region revealed that multiple Ets family members are able to activate the RhoC gene to varying degrees, with Ets-1 and Ets-2 being the most potent regulators (8.2- and 7.5-fold induction, respectively; Figure 2c). This is equivalent to the activation of other Ets-1 responsive promoters in the Flt-1 and urokinase genes (Dube *et al*., 1999). However, we were not able to detect expression of Ets-2 by reverse transcriptase–polymerase chain reaction (RT–PCR) (not shown), confirming our previous assertion that Ets-1 is the predominant member of this protein family that is expressed in the LIM 1863 cells (Bates *et al*., 2003).

Chromatin immunoprecipitation (ChIP) was performed to evaluate whether the Ets-1 protein interacts with specific regions of the RhoC promoter, and to determine if Ets-1 binding is EMT-dependent. Indeed, immunoprecipitation of Ets-1 resulted in the isolation of fragments of DNA corresponding to the RhoC promoter as determined by PCR (Figure 2d). Of the four regions examined, ChIP-2 and ChIP-3 both demonstrated strong evidence of this interaction (Figure 2a and d). Specifically, PCR products corresponding to these two regions were detected in post-EMT but not in pre-EMT cells, while no significant change was observed in either the input or control IP samples. Along with ChIP-4, ChIP-1 showed minimal binding to Ets-1 in post-EMT cells, consistent with the weak transactivation of this region in the promoter assay (Figure 2b and d). While ChIP-1 demonstrates a nominal signal in the post-EMT sample, ChIP-4 did not generate a PCR product in either pre- or post-EMT cells. The involvement of this transcription factor in the regulation of RhoC expression during the EMT of LIM 1863 cells is substantiated by our previous finding that the expression of Ets-1 itself is induced during this EMT (Bates *et al*., 2003).

RhoC enhances and RhoA inhibits post-EMT cell migration

The functional consequences of RhoC activation and suppression of RhoA activation during the EMT of LIM 1863 cells were assessed. For this, a retroviral

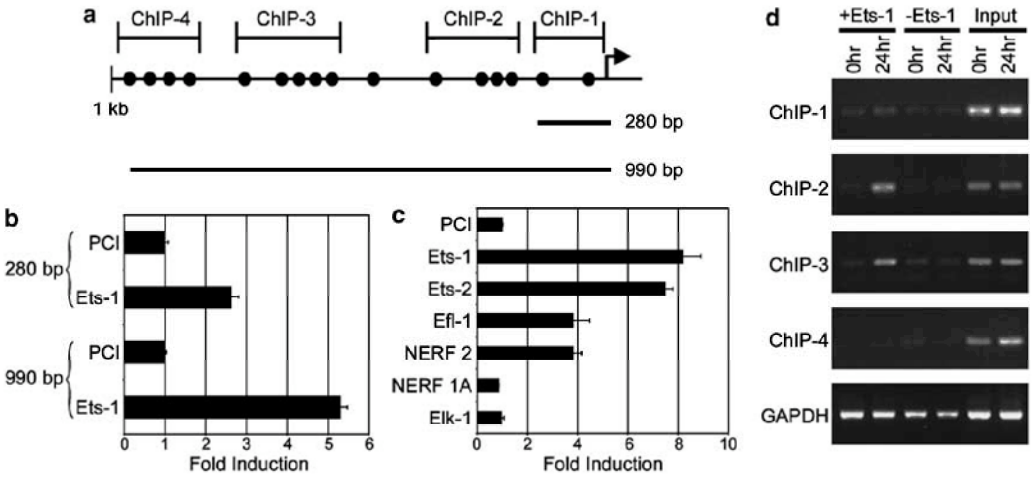

Figure 2 RhoC transcription during EMT is regulated by Ets-1. (**a**) Schematic of the human RhoC promoter. Circles indicate the presence of a putative Ets-1 site (GGAA/T). Promoter constructs corresponding to the regions indicated below were generated in pGL3-Basic. PCR primers were designed for ChIP to amplify the regions above. (**b**) Promoter regions of two lengths (~990 and 280 bp) were tested for the ability of Ets-1 to enhance transcription of a luciferase reporter, standardized against a PCI negative control. The relative increase in luciferase activity was 2.6-fold for the 280 bp and 5.3-fold for the 990 bp construct. (**c**) The larger promoter fragment was tested for the ability of multiple Ets family members to enhance the transcription of a luciferase reporter. Ets-1 activated 8.2-fold, Ets-2 7.5-fold, Efl-1 and NERF 2 3.8-fold compared to PCI control. NERF 1A and Elk-1 demonstrated relatively little induction. (**d**) LIM 1863 cells were treated with cytokine for either 0 or 24 h, and cross-linked chromatin was immunoprecipitated with or without an antibody to Ets-1. Polymerase chain reaction corresponding to the four regions in (**a**) were used. A significant increase in signal was obtained using ChIP-2 and -3 primer sets, but not ChIP-1 or -4. GAPDH was used to detect background DNA contamination. An input control demonstrates relatively equal recovery of DNA from samples.

system to express either RhoA or RhoC shRNAs in the LIM 1863 cells was used. Immunoblots demonstrated that, upon expression of these shRNAs, RhoA protein was reduced in both pre- and post-EMT cells by ~90%, while RhoC expression was reduced by ~95% in the post-EMT cells compared to a scrambled control shRNA (Figure 3a). Reduction of RhoA and RhoC was also observed at the mRNA level (not shown). Interestingly, there appears to be a compensatory increase in RhoA of ~35% in pre- and post-EMT cells upon reduction of RhoC, as well as an increase in RhoC expression of ~50% upon reduction of RhoA in post-EMT cells. Furthermore, the RhoA shRNA caused a reduction in active RhoA of ~65% and the RhoC shRNA resulted in its diminished activation by ~75% in post-EMT cells (Figure 3b). Importantly, shRNA targeted to either RhoA or RhoC was not sufficient to inhibit EMT as determined by loss of E-cadherin expression (Figure 3a) and change in morphology (not shown).

LIM 1863 cells expressing the Rho shRNAs were assayed for their ability to migrate towards conditioned medium from 3T3 cells. Following EMT, cells with reduced RhoA expression were 129% more motile than were cells treated with a scrambled control, while RhoC shRNA resulted in a 54% reduction in cell migration ($P<0.05$; Figure 3c). Notably, even though it has previously been shown that RhoA activity is suppressed during EMT to facilitate migration (Bellovin *et al*., 2005), expression of shRNA for RhoA was able to enhance cell motility further (Figure 3c).

RhoC expression in colon carcinoma serves as a prognostic indicator

The data obtained from the LIM 1863 model suggest that RhoC expression may be associated with more aggressive colon carcinomas. To test this possibility, RhoC expression was assessed by immunohistochemistry (IHC) in tissue microarrays of primary colorectal adenocarcinomas for which clinical outcome was known. Of the 566 tumors analyzed, 29.3% (166 tumors) had no discernible RhoC expression (Figure 4b, Table 1). Conversely, positive staining was detected in 400 tumors (70.7%), though the level of signal did vary considerably. Low expression of RhoC was observed in 198 samples, representing 35% of all tumors examined (Figure 4c, Table 1). Of the remaining tumors, 134 (23.7%) exhibited moderate to high levels of expression, and 68 (12%) demonstrated very high expression (Figure 4d and e, respectively, Table 1). Of note, no expression was detected in normal colon (Figure 4a). To confirm the specificity of the antibody used, IHC was performed on a parallel array without the addition of primary antibody (Figure 4f). In addition, SUM-159 breast carcinoma cells that expressed shRNA for either RhoC or RhoA, or a scrambled control (Simpson *et al*., 2004) were examined by IHC. The control cells and those that expressed the RhoA shRNA exhibited relatively high expression of RhoC (Figure 4g and i, respectively), but staining was diminished significantly in cells expressing the RhoC shRNA (Figure 4h).

Univariate analysis revealed that moderate or high RhoC expression correlates with a significant reduction in patient survival. Specifically, the median survival time for patients with moderate or high RhoC expression were 41.8 and 43.5 months, respectively, compared to 75.5 months for patients with tumors that had no discernible expression (Figure 5, Table 1). Patients with tumors demonstrating low expression of RhoC had a median survival time of 51.4 months (Figure 5, Table 1). The correlation between RhoC expression and survival

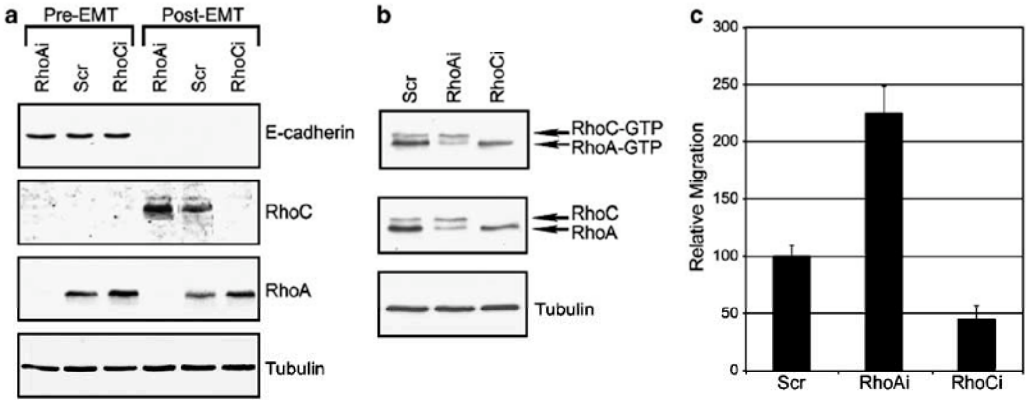

Figure 3 RhoC and RhoA have differing impact on post-EMT cell migration. (**a**) Immunoblot of extracts of LIM 1863 cells stably expressing shRNAs specific for RhoC, RhoA, or a scrambled control. Expression of RhoC is reduced by ~95% in post-EMT cells, while RhoA is reduced by ~95% in pre-EMT and ~90% in post-EMT cells compared to control cells as assessed by densitometry. Loss of Rho protein expression does not affect E-cadherin expression as evidenced by the E-cadherin immunoblot. (**b**) Rhotekin assay for Rho activity. Active, RhoC-GTP and RhoA-GTP (top panel) and total protein (middle panel) are shown. RhoC RNAi cells exhibit a 75% decrease in RhoC activity, and RhoA RNAi cells exhibit a 66% reduction in RhoA activity, as assessed by densitometry. Tubulin immunoblot demonstrates equivalent protein loading (lower panel). (**c**) Chemotactic migration assay. Post-EMT cells that express the RhoC shRNA had a significant reduction in migration (54% ($P<0.05$)), and RhoA shRNA cells demonstrate a significant increase in migration (129% ($P<0.05$)) in comparison to control cells.

was significant ($P=0.0168$). As expected, tumor stage and lymph node status are predictive for patient outcome in our data set, confirming the validity of this analysis. However, while RhoC is a predictor of patient outcome, it does not appear to correlate significantly with either tumor stage or lymph node metastasis. Thus, RhoC expression appears to also be fairly significant in a multivariate setting ($P<0.05$), though lymph node status is still the single best predictor using such an approach ($P<0.001$).

RhoC expression correlates with altered E-cadherin in colon carcinoma

As RhoC expression correlates with E-cadherin loss in the EMT model, it was determined whether such a correlation exists in colon carcinomas using the tissue arrays. Indeed, RhoC expression was associated with either loss or altered localization of E-cadherin. While tumors with no discernible RhoC expression were predominantly associated with membranous E-cadherin (62.1%; Figure 6a and b, Table 2), the majority of

Table 1 RhoC expression in colorectal tumors and associated survival rates

RhoC expression	Tumors	% Total	50% survival (months)	5-Year survival (%)	10-year survival (%)
None	166	29	75.5	54.0	40.5
Low	198	35	51.4	46.6	33.3
Moderate	134	24	41.8	44.1	27.6
High	68	12	43.5	44.8	26.9
All	566	100	53.5	48.0	33.5

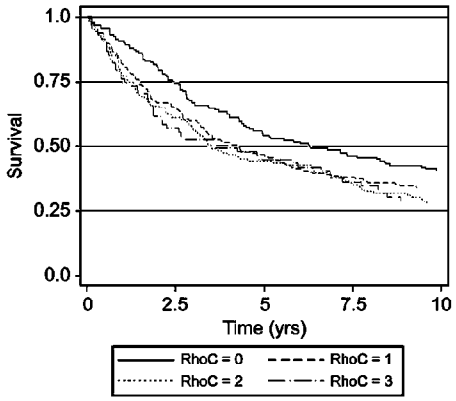

Figure 5 Kaplan–Meier survival analysis of patients as a function of RhoC expression. Samples were organized into four groups based on their level of RhoC expression as defined in Figure 4. The variation in survival as a function of RhoC expression is significant ($P=0.0168$).

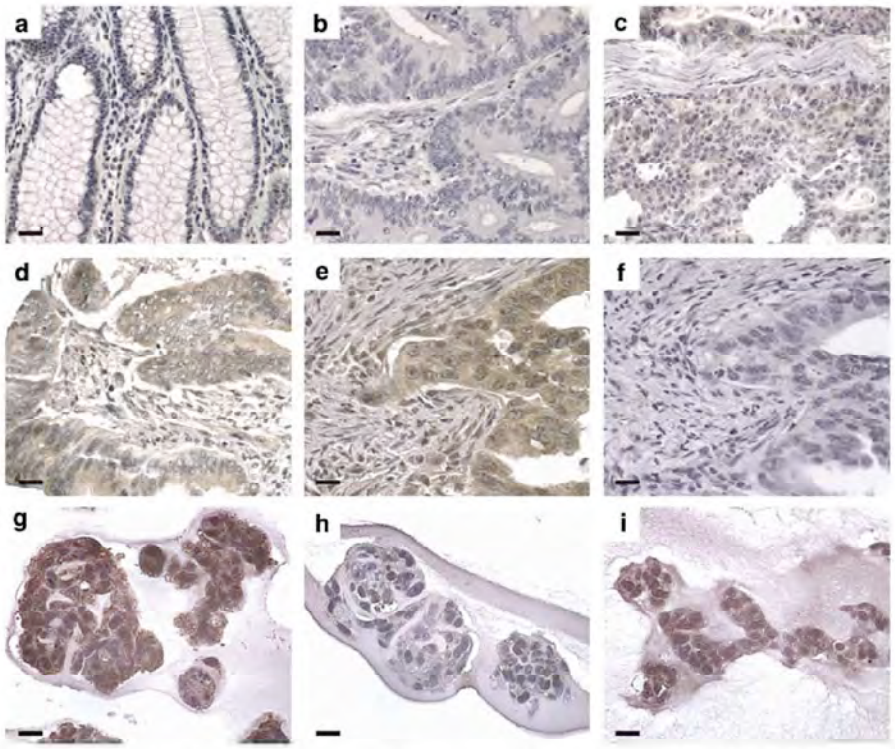

Figure 4 RhoC protein expression in colorectal tumors. The expression of RhoC (**a**–**f**) was evaluated by immunohistochemistry in 566 human colorectal carcinomas. Expression of RhoC was scored as either none (**a**, **b**, **f**), low (**c**), moderate (**d**) or high (**e**). Normal colon tissue (**a**) and a negative control without the addition of primary antibody (**f**) are shown. Specificity of the antibody was confirmed by IHC in SUM-159 cells stably infected with shRNAs targeting either a scrambled control (**g**), RhoC (**h**) or RhoA (**i**). Bar equals 25 μM.

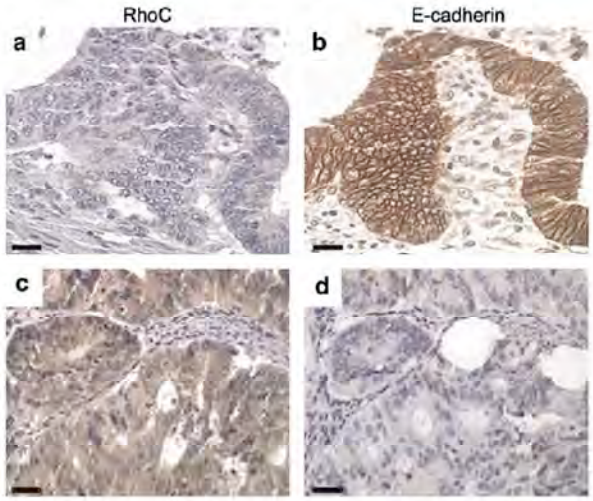

Figure 6 Correlation between RhoC and E-cadherin expression/localization in colorectal tumors. The expression of RhoC (**a**,**c**) and E-cadherin (**b**,**d**) was evaluated by IHC in 555 human colorectal carcinomas. Low or no expression of RhoC (**a**) correlated with membranous localization of E-cadherin (**b**), while high RhoC (**c**) correlated with either loss or delocalization of E-cadherin (**d**). Bar equals 25 μM.

tumors with high RhoC expression had abnormal expression or localization of E-cadherin (75.8%; Figure 6c and d, Table 2). In contrast, abnormal E-cadherin was seen in only 37.9% of tumors with no discernable RhoC expression (Table 2). Tumors with low or moderate RhoC expression corresponded with aberrant E-cadherin in 58.5 and 62.4% of tumors, respectively (Table 2). The correlation between RhoC and E-cadherin expression is statistically significant ($P<0.001$).

Table 2 Association of RhoC expression with altered E-cadherin *in vivo*

RhoC	E-cadherin	
	Membranous (%)	Abnormal (%)
None	62.1	37.9
Low	41.5	58.5
Moderate	37.6	62.4
High	24.2	75.8

Discussion

A function for RhoC in tumor metastasis has been demonstrated in a transgenic model of breast cancer, and it has been inferred from several profiling studies (van Golen *et al*., 1999; Clark *et al*., 2000; Wang *et al*., 2004b; Hakem *et al*., 2005). What has not been examined, however, is the mechanism by which the expression and activation of this GTPase are regulated in cancer. In this study, we provide evidence that RhoC expression and activation can be stimulated as a consequence of the EMT of colon carcinoma, a process that is characterized by loss of E-cadherin and is associated with aggressive disease. Additionally, we identify a specific transcription factor, Ets-1, that regulates RhoC expression during the EMT. Our finding that RhoC expression in a large cohort of tumors is associated with either loss of E-cadherin expression or its delocalization from adherens junctions substantiates the relevance of these data to human colon cancer. Most importantly, RhoC expression may serve as a prognostic indicator for colorectal carcinoma because it correlates with a significant reduction in patient survival time.

Our findings reinforce the hypothesis that the EMT can be a powerful approach for identifying molecules whose expression is associated with aggressive disease and that may serve as potential therapeutic targets. This hypothesis must be considered, however, in light of the current debate on whether a *bona fide* EMT occurs during cancer progression. It has been argued that there is a lack of convincing pathological evidence for EMT in tumors (Tarin *et al*., 2005). However, this argument resides in large part on the specific definition of EMT and whether epithelium and mesenchyme should be considered as cell lineages or general phenotypes (Thompson *et al*., 2005). Supporting the efficacy of EMT as a model for cancer progression, recent studies have shown that molecular markers of EMT *in vitro* are not only present in tumors, but their expression correlates with advanced disease as defined by reduced patient survival, advanced tumor stage and metastasis (Bates *et al*., 2005; Bellovin *et al*., 2005; Moody *et al*., 2005). Moreover, expression of proteins known to induce EMT *in vitro* and during development, including Snail and Twist transcription factors, function as markers and enhancers of carcinoma progression *in vivo* (Yang *et al*., 2004; Roy *et al*., 2005). While these observations do not demonstrate the existence of EMT in carcinoma specifically, they do indicate that key aspects of the EMT can be manifested in carcinomas and that they are associated with more aggressive disease.

The demonstration that multiple pathways intrinsic to EMT are invoked in both cancer and development underscores the relationship between these two biological processes. Such observations indicate that the EMT of carcinoma may involve not only the loss of E-cadherin and expression of mesenchymal proteins, but the neo-expression of proteins that are present during epithelial development, as well. These proteins may also be induced during wound healing and in response to inflammation, pathological processes that can involve an EMT. We have previously shown that the EMT of colon carcinoma involves expression of the β6 integrin, which is expressed in the developing intestine but not in the adult (Bates *et al*., 2005). Indeed, the presence of markers during colorectal tumorigenesis is closely correlated to their embryonic expression and subsequent gene silencing during intestinal development (Hu and Shivdasani, 2005). Hence, the EMT-dependent expression of RhoC and its presence in a sub-population

of tumors suggests that it may behave as an 'onco-fetal' protein. Although data on its expression during embryogenesis are scant, it appears that normal tissue, either epithelial or mesenchymal, lacks significant expression of RhoC. Thus, it will be essential to examine the expression of RhoC during colonic development to substantiate this hypothesis. Interestingly, loss of RhoC does not appear to affect normal murine development, although it remains to be determined whether other members of the Rho GTPase family may be functioning in a compensatory manner upon RhoC loss (Hakem *et al.*, 2005).

Our identification of Ets-1 as a transcription factor that regulates the expression of RhoC provides the first insight into how the expression of this GTPase is regulated in cancer, and it emphasizes the potential importance of Ets-1 in the EMT. The role of Ets-1 in multiple biological processes, including hematopoiesis and angiogenesis, is fairly well documented (Bories *et al.*, 1995; Sato, 1998). Consistent with our data, Ets-1 expression in breast, prostate and other carcinomas is indicative of reduced patient survival (Span *et al.*, 2002; Alipov *et al.*, 2005). Using the LIM 1863 cell line, we have previously reported additional targets of this transcription factor involved in cell migration and survival, including β6 integrin and the VEGF receptor, Flt-1 (Bates *et al.*, 2003, 2005). Moreover, the expression of Ets-1 itself is induced early during the EMT of LIM 1863 cells (Bates *et al.*, 2003). Another link between Ets-1 and the EMT is the finding that the DNA binding ability of Ets transcription factors is regulated by TGFβ-induced acetylation and MAP Kinase phosphorylation, both of which have been shown to be critical for EMT in LIM 1863 cells (Bates and Mercurio, 2003; Tootle and Rebay, 2005). Clearly, studies aimed at understanding how Ets-1 expression and function are regulated during the EMT are warranted and relevant to increasing our understanding of how this transcription factor functions in cancer.

An interesting phenomenon that emerges from our work is the reciprocal relationship between RhoC and RhoA activation that occurs during the EMT and may impact tumor progression. Specifically, we show that RhoC is activated rapidly upon its expression in LIM 1863 cells and that this activation occurs concomitantly with a downregulation of RhoA activity (Figure 1c). These findings are validated by our previous work elucidating a similar relationship between these two proteins in breast cancer (Simpson *et al.*, 2004). Ets-1 provides one mechanism for how RhoC expression is regulated, but another important issue is the mechanism by which RhoA activation is suppressed during the EMT. To this end, we reported recently that p120ctn can associate with RhoA during the EMT of LIM 1863 cells and, as a result, suppress its activation (Bellovin *et al.*, 2005). Our preliminary data, however, suggest that p120ctn has no effect on RhoC activation (data not shown). Nonetheless, it will be informative to assess whether a causal relationship exists between the suppression of RhoA activation and the induction of RhoC expression and activation.

In addition to our findings on the mechanism of RhoC regulation, our data add to an increasing literature on the association of this GTPase with aggressive tumors (van Golen *et al.*, 1999; Clark *et al.*, 2000; Shikada *et al.*, 2003; Wang *et al.*, 2004b; Alipov *et al.*, 2005). Taken together, these findings highlight the feasibility of RhoC as a prognostic marker for aggressive disease. Interestingly, although RhoC expression was negatively correlated with patient outcome, it was not associated with increased tumor stage or metastasis in our analysis of colon carcinomas. This finding indicates that RhoC can be used as a marker of prognosis independently of tumor stage and lymph node metastasis to enhance the predictability of outcome in a multivariate setting. It is also interesting to speculate that RhoC expression may be induced by inflammation based on corroborating reports in other tissues. This possibility is substantiated by the association of RhoC with inflammatory breast cancer (van Golen *et al.*, 2000). Furthermore, it has been shown that RhoC expression is induced in the lung by an inflammatory mechanism that is retractable by glucocorticoid treatment (Schwiebert *et al.*, 1997). Assuming that inflammation can induce RhoC expression in colon carcinoma, this mechanism may help explain the positive effect of dexamethasone treatment on colon cancer (Wang *et al.*, 2004a).

Another novel aspect of our tumor data is that we link RhoC expression in colon carcinomas with alterations in adherens junctions proteins known to be associated with an EMT. Specifically, comparing the level of RhoC to expression and localization of E-cadherin in the tissue array, we demonstrate a positive correlation between increased RhoC expression and dysregulated epithelial junctions in colorectal carcinoma. It is important to note that this relationship with altered E-cadherin is not absolute. While there is significant correlation between the expression of these two molecules, high levels of RhoC can be detected in some tumors expressing junctional E-cadherin, presumably being regulated by an EMT-independent mechanism.

In conclusion, our data provide one mechanism for how RhoC expression is regulated in colon carcinoma, and they substantiate its usefulness as a prognostic marker. These data also validate the EMT as a fruitful approach to understanding mechanisms involved in the genesis of aggressive carcinomas and for identifying potential therapeutic targets. Importantly, however, these findings, as well as those of other recent studies, argue that the EMT in carcinomas may be a more complex process than that which occurs during development, and it may involve aspects of both a mesenchymal transition and epithelial dedifferentiation.

Materials and methods

LIM 1863 Cells and EMT

LIM 1863 human colon carcinoma cells were induced to undergo EMT by treatment with TGF-β1 and TNF-α (R&D Systems, Minneapolis, MN, USA) at 2 and 10 ng/ml,

respectively, and incubation at 37°C. At defined times after cytokine stimulation, cells were harvested and assessed by immunoblot for E-cadherin expression (see below) to monitor the EMT.

Analysis of protein and mRNA expression

To evaluate the expression of specific proteins as a function of the EMT, LIM 1863 cells were stimulated with cytokine for the times indicated and extracted in a Triton X-100 buffer as described previously (Bellovin *et al*., 2005). Immunoblotting was performed using anti-E-cadherin (1:1000, BD Pharmingen, San Diego, CA, USA), anti-RhoA (1:500, Santa Cruz, CA, USA) or anti-tubulin (1:2500, Sigma-Aldrich, St Louis, MO, USA) mAb or with anti-RhoC (1:200, Bethyl Laboratories) polyclonal Ab. The RhoC Ab was generated by inoculating a rabbit with a peptide corresponding to the C-terminal region of the protein. Immune serum was collected and processed over columns containing two peptides each for the homologous regions of RhoA and RhoB. The antibody was then affinity purified on columns containing RhoC peptide and tested by ELISA using the immunizing peptide.

To determine the relative level of RhoC, RhoA and GAPDH (control) mRNA expression during EMT, LIM 1863 cells were stimulated with cytokine for the times indicated, and RNA was extracted and analysed by RT–PCR as described previously (Simpson *et al*., 2004). Primers were as follows: RhoC forward, 5′-ATGCGTGCAATCCGA AAGAAG-3′; RhoC reverse, 5′-TCAGAGAATGGGACAG CCCCT-3′; RhoA forward, 5′-ATGGCTGCCATCCGGAA GAAA-3′; RhoA reverse, 5′-TCACAAGACAAGGCAACCA GA-3′; GAPDH forward, 5′-CCTGGCCAAGGTCATCCAT GAC-3′; GAPDH reverse, 5′-TGTCATACCAGGAAATGA GCTTG-3′.

RNAi

The generation of retroviruses that expressed shRNAs for RhoA and RhoC was described previously (Simpson *et al*., 2004). These retroviruses were added to LIM 1863 cells in the presence of 5 μg/ml polybrene. Following 72 h incubation with virus, cells were placed in low calcium RPMI 1640 supplemented with 5% FCS to generate a single-cell suspension, and 5 μg/ml puromycin was added for 72 h. Selected cells were maintained in normal medium containing 5 μg/ml puromycin. Reduction in target protein expression was determined by immunoblot.

Rho activity

RhoA and RhoC activity was assessed using the Rho-binding domain of Rhotekin (RBD) as described (Ren and Schwartz, 2000; Bellovin *et al*., 2005). Samples were analysed on 15% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) gels followed by immunoblotting using anti-Rho polyclonal antibody, which recognizes both RhoA and RhoC (1:500, Santa Cruz), and anti-rabbit secondary (1:10 000, Pierce, Rockford, IL, USA). Densitometry was performed on the resultant immunoblots using IP Lab Spectrum (BD Biosciences, Rockford, MD, USA).

Migration assays

The ability of LIM 1863 cells to migrate towards 3T3 cell-conditioned medium was performed as described previously (Bellovin *et al*., 2005).

Promoter assays

Two fragments of the RhoC promoter of 277 and 991 nucleotides, corresponding to the regions 234 and 947 nucleotides upstream of the transcription start site, were cloned from human genomic DNA and sub-cloned into the *Sac*I–*Hind*III site of the pGL3-Basic luciferase reporter vector (Promega). Complementary DNA sequences encoding a panel of Ets factors were subcloned into the pCI mammalian expression vector (Promega). Cotransfections of 2.5×10^5 HEK293 cells were carried out with varying amounts of reporter gene construct DNA and expression vector DNA using 4 μl Lipofectamine as previously described. The cells were harvested 16 h after transfection and assayed for luciferase activity. Transfections for every construct were performed independently and in duplicate. Cotransfection of a second plasmid for determination of transfection efficiency was omitted, because potential artifacts with this technique have been reported and because many commonly used viral promoters contain potential binding sites for Ets factors.

ChIP analysis

Chromatin immunoprecipitation (ChIP) was performed using the Chip Assay Kit (Upstate) according to the manufacturer's instructions. Briefly, LIM 1863 cells were treated with TGF-β and TNF-α for 24 h or left untreated, and crosslinking of chromatin was performed with 1% formaldehyde for 10 min at 37°C. Cells were washed twice with ice-cold PBS containing protease inhibitors (aprotinin, pepstatin, leupeptin and phenylmethylsulfonyl fluoride (PMSF)), collected by centrifugation at 4°C, resuspended in 200 μl of SDS lysis buffer, and incubated on ice for 10 min. The lysates were sonicated to shear the DNA to lengths between 250 and 400 bp. For immunoprecipitation, the lysates were precleared for 30 min then incubated with an Ets-1 polyclonal Ab (Santa Cruz Biotechnology Inc.) at 4°C overnight. Negative controls were incubated overnight without primary antibody. Protein A agarose was added for 1 h at 4°C, and beads were washed once with low-salt immune complex buffer, once with high-salt immune complex buffer, once with LiCl immune complex buffer, and twice with Tris-EDTA buffer. The protein–DNA complexes were separated from the antibody by 15 min incubation at room temperature with fresh elution buffer (1% SDS, 0.1 M $NaHCO_3$) with rotation. After centrifugation, the supernatant was collected. Protein–DNA complexes were decrosslinked with 0.2 M NaCl at 65°C for 4 h. DNA was recovered by phenol–chloroform extraction and ethanol precipitation. The following primers corresponding to four regions in the human RhoC promoter were used for PCR: ChIP-1 forward, 5-GCATCTGGGTACCGAAGGA-3; reverse, 5′-GGGTTTCGAGTCAGACTTC-3′; ChIP-2 forward, 5′-ATTACTTGGGGCTTTGGGG-3′; reverse, 5′-TCCTTCG GTACCCAGATGC-3′; ChIP-3 forward, 5′-GAACTGTGC AGATGCTCGC-3′; reverse, 5′-CAGCACAGTGACTGAGA AG-3′; ChIP-4 forward, 5′-CTCCCAGCCCAGACTCTAG-3′; reverse, 5′-GCGAGCATCTGCACAGTTC-3′. 5 μl sample was added to the One-Step RT–PCR kit (Qiagen) containing the indicated primers at a concentration of 0.5 μM. Thirty-five cycles of PCR were performed (94°C melting, 60°C annealing, 72°C polymerizing), and final products were analysed on 1% agarose gels in TAE.

Tissue microarray and IHC

Tissue microarray slides were generated and processed for IHC as described previously (Bellovin *et al*., 2005) using the RhoC polyclonal Ab (1:750, Bethyl Laboratories). Scoring was performed by two observers with review of ~15% initially discrepant cases.

Immunohistochemistry was similarly performed on SUM-159 breast carcinoma cells stably infected with retrovirus for shRNA of RhoC, RhoA, or a scrambled control. Reduction in RhoA and RhoC protein expression has previously been demonstrated by immunoblot (Simpson *et al*., 2004). Wells of a 24-well dish were precoated with 200 μl of undiluted phenol-red free Matrigel (10.2 mg/ml; BD Biosciences). Cells were harvested, washed three times with PBS, and diluted to a concentration of 1×10^4 per well in a volume of 200 μl. Cells were mixed with 100 μl of undiluted ice-cold Matrigel for a ratio 2:1, and laid over the bottom layer. After gelling, complete culture media was added and changed every 2 to 3 days. Morphology was assessed at day 11, and the resulting cell blocks were formalin-fixed, mounted in paraffin, sectioned and treated for IHC as described above.

Statistical methods

Kaplan–Meier method was used to estimate median, 5-year, and 10-year survival along with their 95% confidence intervals. Log-rank test was used to examine the differences in survival between groups. The proportional hazard ratio model was utilized in a multivariate setting, adjusting for the effects of other covariates, including age, gender, tumor stage and lymph node status. χ^2 test was used to determine the significance of association between RhoC expression and E-cadherin.

Acknowledgements

This work was supported by NIH Grant CA107548 (AMM) and DOD Fellowships W81XWH-04-1-0339 (DIB) and W81XWH-04-1-0360 (KJS).

References

Alipov G, Nakayama T, Ito M, Kawai K, Naito S, Nakashima M *et al*. (2005). *Histopathology* **46**: 202–208.
Bates RC, Bellovin DI, Brown C, Maynard E, Wu B, Kawakatsu H *et al*. (2005). *J Clin Invest* **115**: 339–347.
Bates RC, Goldsmith JD, Bachelder RE, Brown C, Shibuya M, Oettgen P *et al*. (2003). *Curr Biol* **13**: 1721–1727.
Bates RC, Mercurio AM. (2003). *Mol Biol Cell* **14**: 1790–1800.
Bellovin DI, Bates RC, Muzikansky A, Rimm DL, Mercurio AM. (2005). *Cancer Res* **65**: 10938–10945.
Bories JC, Willerford DM, Grevin D, Davidson L, Camus A, Martin P *et al*. (1995). *Nature* **377**: 635–638.
Clark EA, Golub TR, Lander ES, Hynes RO. (2000). *Nature* **406**: 532–535.
Dube A, Akbarali Y, Sato TN, Libermann TA, Oettgen P. (1999). *Circ Res* **84**: 1177–1185.
Hakem A, Sanchez-Sweatman O, You-Ten A, Duncan G, Wakeham A, Khokha R *et al*. (2005). *Genes Dev* **19**: 1974–1979.
Hu M, Shivdasani RA. (2005). *Cancer Res* **65**: 8715–8722.
Moody SE, Perez D, Pan TC, Sarkisian CJ, Portocarrero CP, Sterner CJ *et al*. (2005). *Cancer Cell* **8**: 197–209.
Ren XD, Schwartz MA. (2000). *Methods Enzymol* **325**: 264–272.
Roy HK, Smyrk TC, Koetsier J, Victor TA, Wali RK. (2005). *Dig Dis Sci* **50**: 42–46.
Sato Y. (1998). *Hum Cell* **11**: 207–214.
Schwiebert LM, Mooney JL, Van Horn S, Gupta A, Schleimer RP. (1997). *Am J Respir Cell Mol Biol* **17**: 106–113.
Shikada Y, Yoshino I, Okamoto T, Fukuyama S, Kameyama T, Maehara Y. (2003). *Clin Cancer Res* **9**: 5282–5286.
Simpson KJ, Dugan AS, Mercurio AM. (2004). *Cancer Res* **64**: 8694–8701.
Span PN, Manders P, Heuvel JJ, Thomas CM, Bosch RR, Beex LV *et al*. (2002). *Oncogene* **21**: 8506–8509.
Tarin D, Thompson EW, Newgreen DF. (2005). *Cancer Res* **65**: 5996–6000 ; discussion 6000–5991.
Thiery JP. (2002). *Nat Rev Cancer* **2**: 442–454.
Thompson EW, Newgreen DF, Tarin D. (2005). *Cancer Res* **65**: 5991–5995 ; discussion 5995.
Tootle TL, Rebay I. (2005). *Bioessays* **27**: 285–298.
van Golen KL, Davies S, Wu ZF, Wang Y, Bucana CD, Root H *et al*. (1999). *Clin Cancer Res* **5**: 2511–2519.
van Golen KL, Wu ZF, Qiao XT, Bao LW, Merajver SD. (2000). *Cancer Res* **60**: 5832–5838.
Wang H, Li M, Rinehart JJ, Zhang R. (2004a). *Clin Cancer Res* **10**: 1633–1644.
Wang W, Yang LY, Huang GW, Lu WQ, Yang ZL, Yang JQ *et al*. (2004b). *Br J Cancer* **90**: 2349–2355.
Woods DB, Ghysdael J, Owen MJ. (1992). *Nucleic Acids Res* **20**: 699–704.
Yang J, Mani SA, Donaher JL, Ramaswamy S, Itzykson RA, Come C *et al*. (2004). *Cell* **117**: 927–939.